Inhibitory activity of cetuximab on epidermal growth factor receptor mutations in non–small cell lung cancers

Jacqueline F. Doody, Ying Wang, Sheetal N. Patel, Christopher Joynes, Sui Ping Lee, Jason Gerlak, Robin L. Rolser, Yanxia Li, Philipp Steiner, Rajiv Bassi, Dan J. Hicklin, and Yaron R. Hadari

ImClone Systems Incorporated, New York, New York

Abstract

Mutations in the kinase domain of the epidermal growth factor receptor (EGFR) were identified in ~15% of all patients with non–small cell lung cancer (NSCLC). These mutations have been established as an indicator of superior response to gefitinib and erlotinib, small molecule inhibitors of the EGFR kinase domain. Whether these mutations would also render patients more susceptible to treatment with cetuximab (Erbitux), an EGFR-neutralizing antibody, is yet to be determined. In this study, we attempted to evaluate the effect of cetuximab on several NSCLC lines harboring some of the more common EGFR mutations (L858R and delL747-T753insS), as well as the recently identified kinase inhibitor–resistant mutation, T790M. We could show that the kinase activity of the abovementioned EGFR mutants was hindered by cetuximab, as detected by both cell-based phosphorylation and proliferation assays. Interestingly, cetuximab also induced enhanced degradation of the EGFR mutants as compared with the wild-type receptor. Most importantly, cetuximab successfully inhibited the growth of NSCLC lines in xenograft models. These results indicate the promising potential of cetuximab as a regimen for patients with NSCLC bearing these mutations. [Mol Cancer Ther 2007;6(10):2642–51]

Introduction

The epidermal growth factor (EGF) receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinase, which also includes ErbB2, ErbB3, and ErbB4. These receptors play a fundamental role in the control of numerous cellular processes such as growth, proliferation, and survival (1, 2). Activation of EGFR in response to ligand stimulation results in autophosphorylation of several tyrosine residues, which then serve as preferred docking sites for SH2 domain–containing downstream signaling molecules. Ultimately, the activation of the Ras/MAPK and phosphoinositide-3-kinase/Akt cascades leads to cellular proliferation and survival (1, 2).

Overexpression of EGFR is a common hallmark in many cancers, including non–small cell lung cancer (NSCLC), thereby making anti-EGFR therapies attractive for this type of cancer (3). Gefitinib and erlotinib are two small molecule tyrosine kinase inhibitors of the EGFR kinase, used as second- or third-line therapies for patients with advanced NSCLC (4–6). Nonetheless, only 10% of treated patients respond to either drug. Recently, several somatic mutations in the EGFR gene have been identified among patients with better response to gefitinib or erlotinib (7–13). These mutations include both short deletions and point mutations residing in exons 18 to 21 of the EGFR kinase domain. Occasionally, some patients developed resistance to tyrosine kinase inhibitor treatment by acquiring a secondary mutation in the EGFR kinase domain, a T790M point mutation (14).

Cetuximab, an IgG1 monoclonal antibody directed against the extracellular domain of EGFR, blocks receptor activation by interfering with ligand binding (15). This antibody was approved for third-line therapy in combination with irinotecan in advanced metastatic colorectal cancer and as a second- and third-line therapy for patients with squamous cell carcinoma of the head and neck. Moreover, cetuximab is currently being evaluated both as a single agent and in combination therapy in a number of clinical trials in patients with NSCLC. Therefore, it is highly important to determine whether patients with NSCLC, who have a higher incidence of EGFR mutations as compared to patients with colorectal cancer and squamous cell carcinoma of the head and neck (7–13, 16, 17), would be responsive to cetuximab treatment.

In this study, we attempted to explore the effect of cetuximab on the two most frequent EGFR mutations identified in patients with NSCLC, the point mutation L858R and the deletion mutation delL747-T753insS. We also investigated the response of the tyrosine kinase inhibitor–resistant mutation, T790M, to cetuximab. Both in vitro and in vivo analyses of EGFR mutants indicated that cetuximab treatment results in decreased EGFR activity in conjunction with increased EGFR degradation, culminating in a significant reduction in tumor growth.
Materials and Methods

Reagents
Clinical grade cetuximab was produced by the ImClone Systems Incorporated manufacturing facility. EGFR antibody was purchased from UBI, antibodies against mitochondrially activated protein kinase (MAPK), p-MAPK, Akt, p-Akt, ErbB2, and ErbB3 were from Cell Signaling, anti- phosphotyrosine was from Calbiochem, anti-c-Cbl was from BD Biosciences, and anti-actin was from Sigma. The phosphospecific EGFR antibodies Tyr845, Tyr992, and Tyr1045 were purchased from Cell Signaling and Tyr1068, Tyr1086, and Tyr1173 were obtained from Biosource.

Full-length human wild-type as well as the EGFR mutants L858R, T790M, and delL747-P753insS were subcloned into the pcDNA3 (Invitrogen) expression vector. The integrity of all constructs was confirmed by DNA sequence analysis.

Cell Lines and Transfections
NIH-H292, NIH-H1650, and NIH-H1975 NSCLC cell lines were purchased from American Type Culture Collection and propagated in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and 1 mmol/L of sodium pyruvate at 37°C and 5% CO2. Transient transfections of HEK-293 cells were done by using LipofectAMINE 2000 (Invitrogen). Plasmid DNA was transfected into cells (20 ng DNA/5 × 104 cells/ml) in suspension, and following a 30-min incubation at 37°C, 5 × 104 cells/well were plated in 12-well Costar plates. At 24 h posttransfection, cells were treated as described below.

Cell Line Immunoprecipitation and Immunoblotting
To determine IC50s, cells were incubated at 37°C with the indicated concentrations of cetuximab for 2 h prior to stimulation with EGF (100 ng/mL for 10 min) and lysed [lysis buffer: 150 mmol/L NaCl, 50 mmol/L Hepes, 1 mmol/L Na3VO4, and protease inhibitors (pH 7.5)]. For degradation studies, stable transfectants or cell lines were either incubated with 30 nmol/L of cetuximab or 100 ng/mL of EGF for the times indicated before lysing the cells. Immunoprecipitation was done on cell lysates with EGFR antibodies and purified by using protein-A Sepharose beads. For immunoblotting, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the specified antibodies.

Immunofluorescence
NSCLC cells were plated at 1 × 105 cells/mL on an eight-chambered slide overnight in complete RPMI 1640. FITC-labeled cetuximab (5 μg/ml; Pierce Labeling Kit) was added to slides at either 37°C or 4°C for 1.5 h with a further incubation of 30 min with 50 nmol/L of LysoTracker Red (Invitrogen). After washing with PBS, cells were fixed in 4% paraformaldehyde for 30 min. A coverslip was placed on the slide after a second PBS wash using GelMount (Biomeca Corp.).

Cell-Based EGFR Autophosphorylation Assay
A 96-well ELISA plate was coated with 100 μL/well of 1 μg/mL anti-EGFR antibodies, and incubated overnight at 4°C. The anti-EGFR antibodies were prepared in a buffer made with 0.2 mol/L of Na2CO3 and 0.2 mol/L of NaHCO3 adjusted to pH 9.6. Prior to adding cell lysates to the wells, the plates were washed thrice with PBS + 0.1% Tween 20 and blocked by 3% bovine serum albumin in PBS (200 μL for 1 h incubation). After removing the blocking solution, 80 μL of cell lysates were transferred to the wells and incubated for 1 h at 4°C. After incubation, the plate was washed thrice with PBS + 0.1% Tween 20. To detect autophosphorylated EGFR, 100 μL of antiphosphotyrosine antibodies (RC20-HRP; Transduction Laboratories) was added per well (final concentration, 0.5 μg/mL in PBS) and incubated for 1 h. The plate was then washed six times with PBS + 0.1% Tween 20. The enzymatic activity of HRP was detected by adding 50 μL/well of equal amounts of the Kirkegaard & Perry Laboratories substrates A and B. The reaction was stopped by adding 0.1 N of sulfuric acid (50 μL/well) and absorbance was detected at 450 nm.

Cell Proliferation
Growth of the NSCLC cell lines was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Promega). Aliquots of 100 μL of cells were plated at 1 × 104 cells/mL in a 96-well plate overnight in complete RPMI 1640. After 24 h, media was replaced with RPMI 1640 supplemented with 1% fetal bovine serum and increasing concentrations of cetuximab. The cells were incubated for an additional 4 days, followed by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent, and plates were read at 490 nm.

Mouse Xenograft
Seven- to 8-week-old female athymic (nu/nu) mice were purchased from Charles River Laboratories. Mice were housed under pathogen-free conditions in microisolator cages with laboratory food and water available ad libitum. All experiments and procedures were done in accordance with U.S. Department of Agriculture, Department of Health and Human Services, and NIH policies regarding the humane care and use of laboratory animals. NSCLC xenografts were established by s.c. injecting between 5 × 106 and 1 × 107 cells/mouse (depending on the cell line) mixed 1:1 in Matrigel (Collaborative Research Biochemicals) into the left flank of athymic mice. Tumors were allowed to reach 200 to 250 mm3 in size for efficacy studies or 350 to 450 mm3 for mechanism of action studies before randomization into groups of 10 mice/treatment. Cetuximab was administered i.p. at 4 and 40 mg/kg. Caliper measurements were used to calculate tumor volumes using the formula $V = \pi / 6$ (length × width × width). For mechanism of action studies, mice were treated for 6 days followed by harvesting of the tumors. Tumor volumes were analyzed using RM ANOVA in the JMP Statistical Discovery package (v.5.1, SAS Institute, Inc.).

Immunoblotting of Tumor Tissue
Tumor samples were removed 6 days after treatment and snap-frozen. Tumor lysates were prepared in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 100 mmol/L sodium orthovanadate, 1 mmol/L...
EDTA, and protease inhibitor tablets; Roche) using a dounce homogenizer. Protein concentrations were determined using bicinchoninic acid protein assay reagent (Pierce). Lysates were run at 50 μg/lane on 4% to 12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Blots were probed with antibodies to either EGFR or actin.

Results

NSCLC Lines Expressing Wild-type or Mutant EGFR Are Responsive to Cetuximab In vitro

NSCLC patients with mutations in the EGFR gene have a higher response rate to treatment with gefitinib except those expressing the T790M mutation (ref. 14; Supplementary Fig. S1).1 To examine the capability of cetuximab to block the activity of EGFR mutants, the NSCLC cell lines NIH-H1975 (which harbors L858R along with T790M mutations) and NIH-H1650 (which expresses delE746-A750) were compared with the NIH-H292 cell line that expresses wild-type EGFR. Upon EGF stimulation, rapid receptor activation and phosphorylation was detected in both the wild-type (H292) and the deletion EGFR mutant (H1650; Fig. 1A). In contrast, the double EGFR mutant (L858R/T790M), expressed in H1975 cells, displays a moderate constitutive phosphorylation, which is further enhanced in response to ligand stimulation. When these three cell lines were treated with cetuximab, a decrease in receptor phosphorylation in H292 and H1650 cell lines was observed. H1975 cells also displayed reduced EGFR phosphorylation; however, cetuximab had no effect on the constitutive, ligand-independent component of receptor phosphorylation (Fig. 1A; Supplementary Fig. S2).1

Although the EGFR deletion mutant exhibited similar IC₅₀ values as that of the wild-type receptor, a slightly higher value was detected in H1975 cells, expressing the double EGFR point mutations (Fig. 1A; Table 1). The activation of downstream effector molecules of EGFR was also investigated to determine the inhibitory effect of cetuximab on these pathways. The presence of the active form of both Akt and MAPK declined in response to cetuximab treatment among all three tested NSCLC lines, as indicated by a Western blot probed with phosphospecific antibodies (Fig. 1B). However, phosphorylated MAPK levels did not decline in H292 cells treated with 10 ng/mL of EGF and cetuximab, as observed in the other two cell lines (data not shown). Partial inhibition of MAPK phosphorylation was detected in H292 cells only when cells were stimulated with lower concentrations of EGF (1 ng/mL). As EGFR expression levels are elevated in H292 cells compared with

Table 1. IC₅₀ values of cetuximab on NSCLC cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cetuximab IC₅₀ (nmol/L), phosphorylation</th>
<th>Cetuximab IC₅₀ (nmol/L), proliferation (1% fetal bovine serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H292</td>
<td>0.3</td>
<td>0.25 ± 0.07</td>
</tr>
<tr>
<td>H1975</td>
<td>1</td>
<td>1.9 ± 0.23</td>
</tr>
<tr>
<td>H1650</td>
<td>0.3</td>
<td>6.7 ± 1.01</td>
</tr>
</tbody>
</table>

NOTE: The IC₅₀ determination of EGFR phosphorylation was obtained from Fig. 1A. Proliferation IC₅₀ values are the result of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays with standard deviations between three experiments.

Figure 1. Cetuximab inhibits both wild-type and mutant EGFR phosphorylation in NSCLC cell lines. A, NSCLC cell lines H292, H1975, and H1650 were incubated with the indicated concentrations of cetuximab 2 h prior to EGF stimulation (100 ng/mL). Whole cell lysates were subjected to SDS-PAGE and probed with the indicated antibodies. Arrow, EGFR band. B, NSCLC cells were incubated with either 10 ng/mL of EGF (H1975 and H1650 cells) or 1 ng/mL of EGF (H292 cells) for 10 min, 30 nmol/L of cetuximab for 2 h, or a combination of both before lysing cells and immunoblotting using the indicated antibodies.
H1975 and H1650, the inhibitory effects of cetuximab on MAPK activation could be detected only at lower EGF concentrations. As anticipated, H1975 cells had constitutively phosphorylated MAPK and Akt, concurrent with the high basal activity of EGFR. However, as EGF stimulation was required for receptors phosphorylated in H1650 cells, it was surprising to detect phosphorylated Akt and MAPK in this mutant cell line prior to EGF stimulation.

It is well documented that inhibition of wild-type EGFR activity by cetuximab leads to the inhibition of cell proliferation (16, 17). To assess whether the same phenomenon occurs in cells harboring EGFR mutations, the effect of cetuximab on cellular proliferation was determined. The proliferation of H292 cells, expressing wild-type EGFR, was inhibited by cetuximab with an IC50 value of 0.25 nmol/L (Table 1). The H1650 cell line with the deletion mutation showed a 27-fold higher IC50 value (6.7 nmol/L), even though receptor phosphorylation kinetics were comparable between these two cell lines. Lacking a correlation between inhibition of phosphorylation and proliferation in H1650 cells, suggests that proliferation of this cell line is most likely mediated by several different pathways, some of which are EGF-independent. On the other hand, H1975 cells, with the double point mutation, was more sensitive to cetuximab inhibition in the proliferation assay (IC50 = 1.9 nmol/L) compared with H1650 cells, despite its basal ligand-independent receptor phosphorylation. Nevertheless, H1975 cells displayed a slightly higher IC50 value for blocking of proliferation as compared with the wild-type cells. These findings are consistent with the fact that cetuximab fails to affect the ligand-independent input of EGFR in these cells.

**Cetuximab Induces Degradation of EGFR Mutants in NSCLC**

Cetuximab blocks ligand-receptor interactions to further inhibit ligand-induced EGFR dimerization, phosphorylation, and activation (18). In addition to this mechanism of action, it has been shown that cetuximab could also induce the internalization and degradation of EGFR (19). To determine whether a mutation in the EGFR gene would affect cetuximab-induced receptor internalization, and consequently, degradation, we analyzed EGFR localization using immunofluorescence microscopy. We restricted internalization by subjecting cells to 4°C and adding FITC-labeled cetuximab to visualize EGFR. In all three cell lines, cetuximab labeling was largely localized to the plasma membrane (Fig. 2A). When cells were incubated at 37°C to allow receptor internalization, a significant shift from the plasma membrane to lysosomes occurred in H1650 and H1975 cells as evidenced by the colocalization of EGFR with a lysosome-specific dye. In contrast, wild-type EGFR still retained most of the surface staining of the membrane, even after 2 h of incubation with cetuximab as evidenced in H292 cells (Fig. 2A). In order to explore the correlation between receptor internalization and degradation, receptor expression levels were determined in the different NSCLC cells following cetuximab treatment. Only a limited amount of degradation of wild-type receptor was observed in H292 cells, even 72 h post-cetuximab treatment (Fig. 2B and C). However, profound cetuximab-induced degradation of the mutated receptors was detected in both H1975 and H1650 cell lines as early as 24 h posttreatment. Among all three lines, EGF stimulation led to a rapid receptor degradation, indicating that the ubiquitin-mediated degradation machinery was intact and active (Fig. 2B and D).

**Cetuximab Modulates EGFR Phosphorylation Sites**

A major disadvantage of using patient-derived cell lines is the diverse genetic background of each tumor that might influence the sensitivity to cetuximab. To examine the capability of cetuximab to inhibit these mutated receptors within the context of an identical cellular background, L858R, delL747-P753insS, T790M, and wild-type EGFR genes were transiently expressed in HEK-293 cells that endogenously express negligible levels of EGFR (11).

As previously observed among the naturally occurring NSCLC lines, all receptor forms responded to ligand stimulation with increased phosphorylation in the HEK-293 cells. This phosphorylation was abrogated by the addition of cetuximab (Fig. 3A). Some constitutive activation of the L858R form was detected, whereas the T790M receptor had no basal phosphorylation levels. This finding suggests that the ligand-independent EGFR phosphorylation in the NSCLC line H1975, expressing the double EGFR mutant (L858R and T790M) was probably attributable to the L858R and not the T790M mutation. Determinations of IC50 values for cetuximab inhibition of receptor autophosphorylation provided similar values to those obtained for the NSCLC lines (Fig. 3B). With the exception of the L858R point mutation, all values ranged between 0.33 and 0.62 nmol/L, whereas the partially activated L858R mutant had a slightly higher IC50 value of 3 nmol/L. These results reinforce those observed in the NSCLC lines, that cetuximab inhibits ligand-induced phosphorylation of the various EGFR mutants.

EGFR activation was further analyzed by measuring the specific autophosphorylation levels of six different tyrosine residues (Y845, Y992, Y1045, Y1068, Y1086, and Y1173). As shown in Fig. 3C, phosphorylation of all tyrosine residues on the wild-type receptor is EGF-dependent. In contrast, analysis of L858R and delL747-T753insS mutants revealed somewhat unusual activation states. Although some sites on the L858R mutant clearly showed EGF-dependent phosphorylation (Y992, Y1045, Y1086, and Y1173), two tyrosine residues (Y845 and Y1068) were shown to be constitutively phosphorylated. These two sites are considered crucial for receptor activity (Y845) and activation of the Ras/MAPK cascade (Y1068; refs. 1, 2). Constitutive activation of these residues allows this mutant to activate downstream signal transduction pathways in a ligand-independent manner. The altered phosphorylation pattern, therefore, explains the partial inhibitory effect that cetuximab has on the L858R mutant. Autophosphorylation analysis of the delL747-T753insS mutant revealed an impaired autophosphorylation pattern. Four of the tested phosphorylation sites were phosphorylated in an EGF-dependent manner. However,
Figure 2. Cetuximab-induced internalization and degradation of EGFR mutants. A, NSCLC cell lines were incubated with FITC-labeled cetuximab for 2 h at either 37°C or 4°C. LysoTracker Red was added during the final 20 min before fixing cells and adding a coverslip. B, NSCLC cell lines were incubated for the indicated times with either 30 nmol/L of cetuximab or 100 ng/mL of EGF. Cells were then lysed and probed with antibodies against EGFR or actin to ensure equal protein loading. C, the level of EGFR in cetuximab-treated cells in (B) was quantitated by densitometry. D, EGFR levels of EGF-stimulated cells from (B) were quantitated by densitometry.
Figure 3. Cetuximab inhibits wild-type and mutant EGFR phosphorylation in HEK-293–transfected cells. A, HEK-293 cells transfected with either wild-type, L858R, T790M, L858R/T790, or DelL747-753insS mutant EGFR were treated with the indicated concentrations of cetuximab for 2 h. Subsequently, 100 ng/mL of EGF was added for 10 min immediately before lysing the cells. Cells were probed as indicated and IC_{50} values were calculated. B, HEK-293–transfected cells were plated in the presence of the indicated concentrations of cetuximab and subsequently stimulated with 100 ng/mL of EGF. Cell lysates of the treated HEK-293 cells were incubated on anti–EGFR-coated plates and phosphorylated EGFR was visualized by probing with HRP-conjugated antityrosine antibodies. Points, percentage of activity (phosphorylation) of the receptor against increasing amounts of cetuximab; bars, SD (n = 3). C, HEK-293 cells were transfected with the indicated constructs, and stimulated with 100 ng/mL of EGF for 10 min. Following stimulation, cell lysates were probed by the indicated antibodies. D, EGFR-transfected HEK-293 cells were stimulated for 20 min with 100 ng/mL of EGF. Cell lysates were subjected to immunoprecipitation with anti-EGFR antibody and probed with the indicated antibodies.
no phosphorylation of residues Y992 and Y1045 was detected, which are the docking sites for PLCγ and c-Cbl, respectively (20, 21).

The fact that residue Y1045 failed to undergo phosphorylation suggests that c-Cbl could not be recruited to the activated delL747-T753insS receptor. To further confirm the existence of an EGFR/c-Cbl complex, protein extracts were subjected to immunoprecipitation with EGFR antibody, and the resulting Western blot was probed to detect the presence of c-Cbl. As shown in Fig. 3D, complex formation was detected between c-Cbl and the wild-type receptor or the point mutation receptors, but not with the deletion mutant. As expected, a preexisting complex between the constitutively active EGFR mutant, L858R, and c-Cbl was detected, supporting the notion that autophosphorylation of residue Y1045 is indeed crucial for c-Cbl recruitment (Fig. 3D).

**NSCLC Xenografts Respond to Cetuximab Treatment**

Next, the effect of cetuximab on EGFR mutants was tested in vivo. Towards that end, the different NSCLC lines were grafted into athymic mice. Tumors were propagated in mice until they reached 200 mm³. Cetuximab was administered at either 4 or 40 mg/kg twice a week and human IgG was used as a control. H292-derived xenografts, expressing the wild-type EGFR were significantly inhibited at both dosages of cetuximab compared with the control group (Fig. 4A). At 30 days posttreatment, when control animals were sacrificed due to ulceration of the tumors, the size of the tumors in cetuximab-treated animals (180 mm³) had regressed from their original 200 mm³ size, and were significantly smaller than the control group (900 mm³). A similar response was observed in the H1975-derived xenograft, where cetuximab-treated animals had a significant decrease in tumor volume over the control group at 16 days posttreatment when controls were sacrificed (400 versus 1,100 mm³, respectively; Fig. 4B). However, this model failed to show continued tumor regression observed in the H292-derived xenografts, suggesting that H1975 cells became refractory to cetuximab treatment earlier than H292. This may be due to the highly aggressive nature of the H1975-derived xenograft as compared with that of the H292-derived xenograft. The deletion mutant NSCLC H1650 also showed a response to cetuximab treatment when implanted into mice, albeit not to the same extent as with the other two NSCLC xenografts (Fig. 4C). These results correlate well with the in vitro cell–based proliferation data (Table 1), indicating that growth of H1650 cells is most likely mediated by EGF-independent pathways.

**EGFR Characterization upon Cetuximab Treatment**

As activation of EGFR could also be mediated via heterodimerization with other ErbB family members, we verified the expression levels of other ErbB receptors among the tested NSCLC. ErbB2 was found to be equally expressed in all cell lines, suggesting that its expression does not contribute to the differences observed between the NSCLC-derived xenograft studies (Fig. 5A). The expression of ErbB3, in contrast, was higher in H1975 cells than in the other two cell lines. However, the xenograft profile of H1975 and H292 was fairly similar, indicating that the differences in ErbB3 expression did not influence EGFR signaling in these cells (Fig. 4). These data agree with those seen in squamous cell carcinomas of the head and neck, in
which no correlation was observed between response to cetuximab and expression of ErbB family members (22).

In light of the in vivo efficacy data and the in vitro degradation finding, the capability of cetuximab to induce receptor degradation in NSCLC xenografted tumors was determined. NSCLC-derived tumors were excised 6 days after the onset of cetuximab treatment. Cellular proteins were extracted, subjected to SDS-PAGE and Western immunoblot to detect EGFR expression (Fig. 5B and C). H292-derived tumors treated with cetuximab displayed a minor decrease in wild-type EGFR levels when compared with controls. When cetuximab-treated H1974-derived tumors expressing the L858R and T790M point mutations, were tested for the expression of EGFR, a more significant decrease in EGFR levels was detected when compared with that of the control group. The difference was even more profound in the H1650-derived xenograft. These results indicate that EGFR degradation could be detected not only in vitro but also takes place in mouse xenograft models, supporting the notion that the mutant EGFR forms were more susceptible to cetuximab-induced degradation than the wild-type receptors.

Discussion

In this study, we analyzed the effects of cetuximab on several EGFR mutants. We showed that the antibody inhibits the phosphorylation and activation of wild-type EGFR as well as that of several receptor mutants in NSCLC lines. In addition, the data clearly indicate that cetuximab is capable of inducing a more pronounced internalization and degradation of EGFR mutants than that of wild-type EGFR. Consequently, this rather unique EGFR down-regulation in response to cetuximab treatment leads to the inhibition of tumor growth when NSCLC lines are grafted in animals.

Analysis of the L858R EGFR point mutation revealed two tyrosine residues which were predominantly phosphorylated in an EGF-independent manner (Y845 and Y1068), whereas the others were phosphorylated in an EGF-dependent manner. Surprisingly, however, even though the L858R EGFR mutant possesses some constitutive activation that could not be inhibited by cetuximab, growth inhibition of the NSCLC line, H1975, harboring this mutation, was detected both in vitro and in vivo. Increased receptor degradation and possibly antibody-dependent cell-mediated cytotoxicity could account for this increase in in vivo activity (19, 23). The gefitinib- and erlotinib-resistant mutation, T790M (14, 24), was found to be sensitive to cetuximab inhibition. Apparently, the threonine residue at position T790, located in the ATP-binding pocket of the kinase domain, is required to form a critical hydrogen bond with gefitinib (25), making this mutant specifically resistant to quinazoline inhibitors. By binding to the extracellular domain of EGFR, cetuximab could inhibit the activity of the T790M mutant with comparable efficiency to that of the wild-type receptor. Therefore, patients expressing the T790M point mutation, which are refractory to gefitinib or erlotinib, may benefit from cetuximab therapy.

Cetuximab was also shown to inhibit the autophosphorylation and activation of the EGFR deletion mutant delL747-T753insS, even though ligand-dependent phosphorylation of two tyrosine residues (Y992 and Y1045) could not be detected in this mutant. It has been postulated that this deletion mutant could induce a dramatic conformational change, affecting the ATP binding cleft (7), thereby limiting the access of activated kinase to tyrosine residues Y992 and Y1045. However, the limited phosphorylation of the receptor does not hinder inhibition by cetuximab. The findings described herein contradict two
Effect of Cetuximab on EGFR Mutants in NSCLC

recent publications (26, 27) in which phosphorylation of several EGFR deletion mutants was not inhibited by cetuximab, even though inhibition of proliferation was detected. The discrepancy in the results could be attributed to the cellular context of the different NSCLC lines, where each cell line may harbor additional mutations that could affect the overall outcome. Mukohara et al. (27) went further and stably transfected NIH3T3 cells with both the L858R and the deletion EGFR mutants, and found that the mutants were refractory to cetuximab treatment. However, these NIH3T3-transfected lines express a very high level of endogenous EGFR that results in constitutive autophosphorylation of the receptors. Because cetuximab is incapable of affecting ligand-independent phosphorylation, the antibody would be ineffectual in this system.

Our data indicate that EGFR mutants are internalized and degraded in response to cetuximab treatment, whereas wild-type receptors seem to be more resistant to this down-regulation, similar to what has been previously reported (19). This cetuximab-induced degradation of mutated EGFR is not contingent on EGFR expression levels because the highest EGFR levels are detected in the H292 cells, expressing wild-type receptor. More likely, the mutated EGFR is unstable, making it more prone to degradation, as suggested by others (28, 29). Apparently, the observed cetuximab-induced degradation of the tested EGFR mutants is mediated by a mechanism other than that of EGF-induced degradation. Several pieces of data support this idea. First, degradation of the mutated receptors in response to cetuximab treatment occurred over a 24-h time period, whereas EGF-induced degradation was substantially shorter. Second, both the phosphorylation of tyrosine Y1045 and formation of the c-Cbl/EGFR complex could not be detected in the dell747-T753insS mutant. Third, EGFR containing a Y1045 mutation, which does not allow c-Cbl binding, is still down-regulated in the presence of an EGFR monoclonal antibody (30).

Inhibition of tumor growth was observed in all three NSCLC lines when tested in tumor xenograft models. In view of the in vitro proliferation data, the anticipated response of the H1650 model to cetuximab was somewhat inadequate. Even though this line expresses higher levels of EGFR than the responsive H1975 line, the differences in the genetic background of this particular cell line may render it less “addictive” to EGF stimulation. Indeed, the high levels of constitutive, ligand-independent phosphorylated MAPK and Akt observed in the H1650 line support this hypothesis. An alternative, EGF-independent pathway possibly activates these two signaling pathways, bypassing the dependency on EGF stimulation for cell growth. Moreover, it has been shown that cetuximab can inhibit the growth of the NSCLC HCC-827 cell line that expresses the same deletion mutation as H1650 (31). Taken together, our results indicate that cetuximab inhibits EGFR mutants in NSCLC by more than one mechanism: (a) blocking ligand-receptor interactions that lead to the inhibition of receptor auto-phosphorylation and activation, and (b) inducing the degradation of EGFR mutants. Therefore, the existence of mutant receptors should not limit a patient’s response to cetuximab. On the contrary, those patients may benefit from therapy because EGFR degradation will enhance the inhibitory effects of cetuximab on the receptor.

References


Molecular Cancer Therapeutics

Inhibitory activity of cetuximab on epidermal growth factor receptor mutations in non–small cell lung cancers

Jacqueline F. Doody, Ying Wang, Sheetal N. Patel, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-06-0506

Cited articles
This article cites 31 articles, 18 of which you can access for free at:
http://mct.aacrjournals.org/content/6/10/2642.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/6/10/2642.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.